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# Evaluation of probiotic potentials of *Lactococcus lactis* sp. and its antimicrobial activity against some food-borne pathogens

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## ABSTRACT

Probiotics are usually used in functional foods due to their nutraceutical effects. But before using a strain to formulate functional foods, many tests have to be done to check some probiotic properties. *Lactococcus lactis* sp (*L. lactis* sp) strain isolated from fermented maize beverage "Sha'a" used in this study was grown in M17 broth for 56 h to determine the growth curve. Tolerance to the extreme gastrointestinal conditions (pH 2.0 and bile tolerance to 3.0%) was studied after gradual adaptation of the culture. Antibiotic resistance and antioxidant activity were evaluated. The ability to inhibit the growth of harmful bacteria was also checked. Results showed that culture strain could resist pH 2.0 and 3% bile for 24 hours. *L. lactis* sp. was able to produce acid which decreased the pH of the medium. Higher antioxidant activity was observed after 18 h of incubation (47.76 ± 0.00%). The strain was susceptible of most the antibiotics tested. Cultures presented a strong inhibition activity against the food-borne pathogens tested (*Citrobacter* sp., *Shigella* sp., *Shigella dysenteriae, Enterococcus feacalis, and Pseudomonas* sp.).

**Key words:** *Lactococcus lactis* sp., acid and bile salt tolerances, antibiotic resistance, antioxidant activity, antimicrobial activity.

## 1. INTRODUCTION

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Probiotics are a group of bacteria that are Generally Recognized As Safe (GRAS) (FAO/WHO, 2002). The uses of probiotics have been shown to confer many health benefits to humans and to play a key role in normal digestive processes, and in maintaining the animal's health (Song et al., 2012). A number of health benefits of probiotics include the balancing intestinal microflora, antidiarrheal properties, reduction in serum cholesterol, reduction of fecal enzymes, improvement in lactose metabolism, enhancement of immune system response, anticarcinogenic properties, improved



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bioavailability of nutrients, antimicrobial activity, enhancement of bowel motility/relief from constipation, improvement in inflammatory bowel disease, and suppression of *Helicobacter pylori* infection in the stomach (Mercenier et al., 2003; Reid et al., 2003; Gill and Guarner, 2004; Jin and Lee, 2009).

For a probiotic to exert a benefit effect on human health it must have good technological properties such as:

(i) Resistance to gastric acidity (low pH),
(ii) Resistance to pancreatic juices, intestinal enzymes,
(iii) Resistance to high bile salts concentration,
(iv) Adherence to the enterocytes through its ability to reduce pathogen adhesion to surfaces,
(v) Antimicrobial activity against potentially pathogenic bacteria (acid and bacteriocin production).

Some microorganisms are very sensitive to gastrointestinal conditions, and their survival during the gastrointestinal transit is a challenge**.** Nowadays, the use of probiotics for technological end, the search for lactic acid bacteria with very good technological properties remains a priority for microbiologists. Besides probiotics continue to surprise us since the discovery of new interesting properties is frequently observed; so microbiologists are still trying to explore new strains or even those already known but isolated in different matrices. *L. lactis* strains are the most widely used starter cultures in the production of cheese (Cavanagh et al., 2014). Some few selected strains are predominantly used as primary starters due to the technological attributes that they possess (Marshall, 1991). The objective of this work was to screen *in-vitro* some probiotic properties of *L. lactis* sp. strain isolated from fermented beverage "Sha'a" of Cameroon; specifically to study the capacity to produce lactic acid, tolerance to low pH and high bile salt as that of GIT conditions, antibiotic properties, antioxidant activity and antimicrobial activity.

## 2. MATERIALS AND METHODS

**Starter**

The starter that was used was the stock culture of *L. lactis* sp. of the Laboratory of Biochemistry, Medicinal Plant, Nutrition and Food science (LAPMAN) of the University of Dschang-Cameroon; isolated from fermented maize beverage "sha'a" of Cameroon.

**Culture media**

Culture media used for lactic acid bacteria growth and cells count were M17 broth and agar respectively. Brain-heart infusion (BHI) broth and agar were used like growth medium to culture pathogenic microorganisms.

**Resistance to low pH**

Lactic acid bacteria isolates obtained from overnight culture were obtained by centrifugation for 10 min at 5,000 rpm and 4 °C, washed twice with PBS buffer (pH 7.2), and adjusted to pH 2.5. Resistance was assessed in triplicates in terms of viable colony counts and enumerated on M17 agar after incubation at 37 °C for 0, 1, 2 and 3 h; indicating the time spent by food in the stomach (Maragkoudakisa et al., 2006; Zoumpoopoulou et al., 2008).

**Resistance to bile salt**

Adaptation against bile salt was carried out based on the intestinal bile at different concentration 0.5%, 1.0%, 2%, 3% (w/v) and during 24 h. M17 medium containing different bile concentration was inoculated with overnight culture of lactic acid bacteria, and viable colonies were counted after 24 h.

**Antibiotic resistance**

Bacteria strain was assessed for antibiotic resistance by disc diffusion method using antibiotics discs. One milliliter of actively growing cultures was mixed with 10 ml of M17 agar and poured into a petriplates. After solidification, the antibiotic disks were placed on the solidified agar surface, and the plates were left over for 30 min at 4 °C for diffusion of antibiotics and then, anaerobically incubated at 37 °C for 48 h. Resistance was defined according to the disc diffusion method by using three different antibiotic discs (Hi Media Laboratories Pvt. Ltd. Mumbai, India.), (Felten et al., 1999). The zone of inhibition was measured in millimeters.

**Antioxidant activity**

***Preparation of intracellular cell free extracts***

*L. lactis* sp. were grown in 50 ml of M17 broth at 37 °C for 0 , 18, 36, 48 h, and harvested by centrifugation using REMI cooling centrifuge, model Remi instruments LTD. VASAI - 401 208 (India) at 8,000 rpm for 20 min at 4 °C. The supernatant was decanted.



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Cells were washed three times with saline 0.9 % by centrifugation at 8,000 rpm for 10 min at 4 °C, and the pellet was taken. Pellet was re-suspended in 2 ml of phosphate buffer (0.2 M; pH 7.0) and cell disruption was carried out with mortar and pestle on ice for 15 min. Cell debris were removed by centrifugation at 8,000 rpm for 10 min at 4 °C, and the resulting supernatant was the intracellular cell free extract. The extract was used for antioxidant activity assay.

***Assay for Antioxidant activity***

The antioxidant activity was measured using the DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging assay according to the method reported by Rubens and Wagner (2004) with some modifications. 1 ml of freshly prepared DPPH solution (5 mg/100 ml methanol) was added to 1 ml of the intracellular cell-free extract, wrapped in aluminum foil, vortexed and kept at room temperature for 30 min in darkness. Control was prepared using 1 ml of phosphate buffer to 1 ml DPPH solution. The blank was taken as phosphate buffer (0.2 M; pH 7.0) and no sample was added. Ascorbic acid (100 µg/ml) was used as synthetic and natural standards. The decrease in optical density was measured under dim light 517 nm. The percentage antioxidant activity was defined as follows:

$$\text{Antioxidant activity (\%)} = \frac{A_0 - A}{A_0} \times 100$$

Where $A_0$= Optical density in control, A= optical density of intracellular cell free extract.

**Antimicrobial activity**

The search for possible production of inhibiting substances by this bacteria strain was carried out using the agar well diffusion technique (Mante et al., 2003). The filtered supernatant and pellets of *Lactobacilli* were applied for screening of antimicrobial activity. The plates were kept at 4°C for 30 min to permit diffusion in the assay material, and incubated at 37°C for 24 h. Isolates were screened for production of antimicrobial against *Shigella*, *Shigella dysenteria*, *Enterococcus faecalis*, *Pseudomonas* and *Citrobacter*. The diameters of the growth inhibition zones were measured and recorded in millimetre (mm).

Bacteria strain was cultivated in M17 broth and incubated at 37 °C during 18 h. After growth, the culture was centrifuged at 8,000 rpm during 10 min, and the supernatant was collected and stored at 4 °C. The cold supernatant fluid was sterilized and filtered with a 0.22 µm pore size filter (Millipore Corporation, Bedford, USA) and adjusted to pH 6.0 with sterilized 2 M NaOH to the supernatants to raise their pH to 6.8 ± 0.1, so as to rule out inhibition through the production of organic acids. These wells received 50, 100 µl of tested supernatant strains and then incubated at 37 °C during 24 h. Inhibition of growth was determined by an area of inhibition surrounding each agar well (Elaine et al., 1994).

**Statistical analysis**

Data collected in triplicate were subjected to statistical analysis. To compare values, analysis of variance (ANOVA) using Microsoft Office Excel (Microsoft, Redmond, WA, USA) was used. Pair-comparison of treatment means was obtained by Tukey's procedure at $P < 0.05$, using the Statistical software for Windows (GraphPad InStat).

## 3. RESULTS AND DISCUSSION

**Adaptation to low pH**

The study of the prolong exposure of this strain with similar conditions to those of the stomach (Table 1) shows the existence of a continuing viability of individual strain decreasing with time (from 0 to 24 h). *L. lactis* sp. showed high resistance to pH 3.0 with survival percentages > 88% even after 24 h of incubation and low resistance at pH 2.0 with 16.24% which can be considered a severe treatment. Mishra and Prasad (2005) also observed that LAB showed higher survival rate at a high pH (3.0) than at pH 2. The presence of viable cells after 24 h at pH 2.0 may be due to gradual adaptation of the strain from high to low pH. These results look similar to those found by Lei et al. (2015) who have reported a decrease in viable cells at pH 2.0 after 3 h of five enterococcal strains whereas Pieniz et al. (2014) had observed that there was no viable *Enterococcus durans* strain LAB18s in the media at pH 2.0 after 1 h of incubation. This indicates that the isolate *E. durans* LAB18s was not resistant to simulated gastric juice at pH 2. In fact before reaching the gastrointestinal tract, probiotic bacteria must first survive transiting through the stomach and exert their health promoting effects as metabolically viable active cells when they arrive in the colon (Dunne et al., 2001).



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**Table 1** Survival of *Lactoccus lactis* sp. strains under acidic and high-bile salts conditions after 3 h and 24 h of incubation at 37°C

| | **pH 4.0** | | | **pH 3.0** | | | **pH 2.0** | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 0 | 3 | 24 | 0 | 3 | 24 | 0 | 3 | 24 |
| Number of cells (log10 CFU/ml) | 8.26 | 6.30 | 5.40 | 5.40 | 5.15 | 4.79 | 4.79 | 3.11 | 0.78 |
| Survival percentage (%) | / | 75.90 | 65.37 | / | 95.37 | 88.78 | / | 64.93 | 16.24 |

**Adaptation to bile salt**

The result to bile salt tolerance test is shown in table 2. The survival rates of the strains decreased with increasing bile salt concentration; but the strain could survive under 3% bile salt (1 log10 CFU/ml) for a long time (24 h). These results are similar to those reported by Lei et al. (2015) where five enterococcal strain were able to survive (>$10^4$ CFU/ml) at high bile salt concentration for 24 h. This strain, however, showed stronger bile tolerance at 3.0% bile concentration than those reported by Papamanoli et al. (2003) who found that Lb. *plantarum* strains and Lb. *curvatus* strains isolates from Greek dry-fermented sausages were resistant to 0.3% bile salts. Pieniz et al. (2014) found that E. *durans* LAB18s was able to survive at all bile salt concentrations tested (up to 1.5%) to give an exponential growth from the inoculation (0 h) until 4 hrs of incubation. In another study, the bile resistance of *L. acidophilus* NIT was evaluated by supplementation with bile (oxgall) in MRS broth containing 1%, 2%, 3% bile. With increase in bile, the growth was obviously decreased. However, it still had more than $10^5$ CFU/ml viable bacteria in MRS broth with 2% bile after 15 h incubation (Pan et al., 2009). Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al., 1992; Taranto et al., 2006). It has been reported that good bile tolerance benefits the colonization in the host GI tract (Luo et al., 2012).

**Table 2** Adaption to different bile salt concentrations

| | **Bile salt concentration (%)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.5 | | 1.0 | | 2.0 | | 3.0 | |
| Time (h) | 0 | 24 | 0 | 24 | 0 | 24 | 0 | 24 |
| Number of cells (log10 CFU/ml | 8.26 | 6.66 | 6.66 | 4.72 | 4.72 | 2.48 | 2.48 | 1 |
| Survival percentage (%) | / | 80.71 | / | 71 | / | 52.43 | / | 40.37 |

**Antibiotic susceptibility**

The diameters of inhibition zones obtained for antibiotic susceptibility of the *L. lactis* sp are shown in table 3. Results indicated that *L. lactis* sp. was susceptible to most of antibiotics tested. This strain was sensitive to vancomycin. It is well known that vancomycin is an antibiotic that belongs to glycopeptide antibiotics inhibiting the peptidoglycan synthesis which is an important structural component of the bacterial cell wall. Therefore, Gram-positive bacteria, including lactic acid bacteria are especially vulnerable to vancomycin treatment (Reynolds, 1989). This result does not confirm the finding of Salimen and Wright (1998) who reported the vancomycin resistance of lactobacilli and concluded that vancomycin resistance is an intrinsic property of lactobacilli. This same susceptibility may be of a disadvantage; however, if the host takes orally administered antibiotics which may eventually eliminate established probiotic LAB.

Resistance cases were observed with three antibiotics such as Co-Trimoxazole, Tetracycline, Aztreonam. Menad et al. (2014) reported that *Lactococcus lactis sbsp cremoris* was sensitive to Tetracycline (TET) and resistant to Penicillin (P) whereas in these results, the strain was resistant to Tetracycline and sensitive to Penicillin, this may be due to the original matrix where our strains have been isolated. This antibiotic resistance may be a problem as it can be transmitted to pathogens in which therapeutic resistance



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could have adverse consequences. The European Food Safety Authority suggests that probiotics may not have acquired resistance to antibiotics (Zago et al., 2011).

**Table 3** Antibiotic susceptibility test of *Lactococcus lactis* sp.

| Antibiotic | Symbol | Concentration | Inhibition zone (mm) |
|---|---|---|---|
| **OD 020 – 1 PK, Octadisc Combi-1** | | | |
| Cephalothin | CEP | 30 mcg | 20 ± 0.00 |
| Clindamycin | CD | 2 mcg | 20 ± 0.00 |
| Co-Trimoxazole | COT | 25 mcg | R |
| Erythromycin | E | 15 mcg | 16 ± 2.83 |
| Gentamicin | GEN | 10 mcg | 18.5 ± 0.71 |
| Oflaxacin | OF | 1 mcg | 20.5 ± 0.71 |
| Penicillin-G | P | 10 units | 28 ± 2.83 |
| Vancomycin | VA | 30 mcg | 20 ± 0.00 |
| **OD 271 – 1 PK, Octadisc Combi 69** | | | |
| Ciprofloxacin | CIP | 5 mcg | 25. 5 ± 3.53 |
| Ofloxacin | OF | 5 mcg | 21 ± 1.41 |
| Sparfloxacin | SPX | 5 mcg | 24 ± 2.83 |
| Gatifloxacin | GAT | 5 mcg | 21 ± 1.41 |
| Aztreonam | AT | 30 mcg | R |
| Azithromycin | AZM | 15 mcg | 13 ± 1.41 |
| Vancomycin | VA | 30 mcg | 21 ± 1.41 |
| Doxycycline | DO | 30 mcg | 24 ± 2.83 |
| **OD 026 – 1 PK Octadisc Combi VII** | | | |
| Amoxicillin | AMX | 10 mcg | 28 ± 2.83 |
| Cloxacillin | COX | 5 mcg | 17 ± 4.24 |
| Erythromycin | E | 15 mcg | 18 ± 0.00 |
| Tetracycline | TE | 10 mcg | R |
| Penicillin | P | 2 units | 22 ± 2.83 |
| Co-Trimoxazole | COT | 25 mcg | R |
| Penicillin – V | PV | 3 mcg | 26 ± 2.83 |
| Cephalexin | CN | 30 mcg | 17.5 ± 0.71 |

Values represent the mean ± STDV of three independent experiments.

**Antioxidant activity**

Probiotic metabolic activities may have an antioxidant effect via the scavenging of oxidant compounds, or the prevention of their generation in the intestine (Azcárate-Peril et al., 2011). We observed that the antioxidant activity increased during the growth of *L. lactis* sp. from 29.85 ± 0.02% at 0 h to 47.76 ± 0.00% after 18 h of incubation. This activity starts to decrease after 18 h from 44.77 ± 0.03% at 36h to reach 42.39 ± 0.01% after 48 h. This means that cell-free extracts of this bacteria strain exhibited antioxidant properties. Antioxidant activity exhibition may be due to the fact that most living organisms possess enzymatic and non-enzymatic antioxidant defense, and repair systems that have evolved to protect them against oxidative stress (Li et al., 2012). These results were higher compared to those reported by Zhang et al. (2011) who have evaluated the antioxidative effect of intact cells and cell-free extract of *Lactobacillus casei subsp. casei* SY13 and *Lactobacillus delbrueckii subsp. bulgaricus* LJJ, isolated from the traditional yogurt by the same method. The intact cells of SY13 and LJJ exhibited the scavenging DPPH radical by 27.50 and 23.99%, respectively. Kanno et al. (2012) also reported that *Lactobacillus plantarum* 7FM10 isolated from the traditional Japanese food narezushi exhibited DPPH scavenging capacity. The selection of specific strains and the evidence of their effectiveness, resulting in control of reactive



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radicals, can be exploited to formulate novel probiotic foods or supplements that can exert a role in the prevention of oxidative stress and related diseases (Ameritti et al., 2012).

**Table 4** Antimicrobial activity using the culture supernatant and *Lactococcus lactis* sp. Cells

| | Diameter of inhibition zones (mm) | | | |
|---|---|---|---|---|
| | Supernatant (µl) | | Pellet (µl) | |
| Pathogens | 50 | 100 | 50 | 100 |
| *Citrobacter* | 12 ± 2.0 | 16 ± 1.0 | 12 ± 1.5 | 14 ± 1.0 |
| *Shigella dysenteriae* | 20 ± 0.5 | 22 ± 1.0 | 18 ± 1.5 | 20 ± 2 |
| *E. feacalis* | 18 ± 1.0 | 20 ± 1.5 | 22 ± 1.0 | 25 ± 1.5 |
| *Pseudomonas* | 15 ± 0.5 | 17 ± 0.5 | 14 ± 1.0 | 16 ± 0.5 |

Values represent the mean ± STDV of three independent experiments.

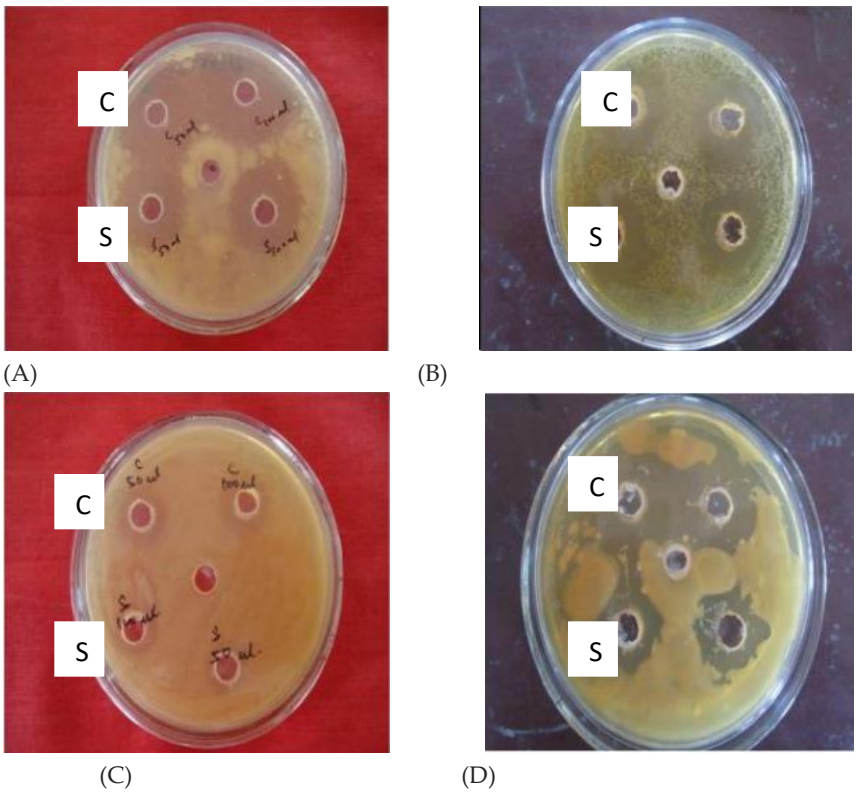


**Figure 1** Inhibition zone of *Lactococcus lactis* sp. cells and supernatant against *Shigella dysenteriae* (A), *E feacalis* (B), *Pseudomonas sp.* (C) and *Citrobacter sp.* (D)

**Antimicrobial activity**

Diameters of inhibition zones of *L. lactis* sp. against *Citrobacter*, *Shigella*, *Shigella dysenteria*, *E. feacalis* and *Pseudomonas* are shown in table 4 and figure 1. The presence of diameter inhibition proved that this strain can inhibit the growth of these five pathogens. The highest inhibitory activity using the intracellular extract was observed against *Shigella* followed by *E.feacalis*. When the culture supernatant was tested, it was observed that the highest antimicrobial activity observed was against *Shigella dysenteriae* (20 ± 0.5 mm). In this study the neutralized supernatant has exhibited an antimicrobial activity, this suggests that *L. lactis* sp. strain may produce bacteriocin. These results are similar to those reported by Pieniz et al. (2014) who had study the antimicrobial activity of the supernatant and intracellular extract of E. durans LAB18s against *L. monocytogenes, E. coli, B. cereus, S. aureus, S. Typhimurium, S. Enteritidis, P. aeruginosa, A. hydrophila, and C. fimi.* They noted a positive antimicrobial activity in both culture supernatant and intracellular extract, against the indicator microorganisms. Menad et al. (2014) evaluated the antibacterial activity of *Lactococcus*



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*lactis sbsp cremoris* against *Salmonella sp* and they observed the presence of an inhibition well zone of 10 mm. Charlier et al., 2009 showed that *Lactococcus sp* has a broad-spectrum inhibition against *Salmonella sp* which is induced by the effect of lactic acid and bacteriocins.

## 4. CONCLUSION

According to the *in-vitro* evaluation of potential probiotic properties of *L. lactis* sp., the strain is able to tolerate low pH and high bile salt as that of GIT conditions, and shows its potential to survive in harsh gut environment. The cell-free extract of this strain exhibited a good antioxidant property after 18 h. *L. lactis* sp. also exhibited antimicrobial activity against many toxic food-borne pathogenic disease causing bacteria. This strain could be collected at the exponential phase (18 h), and used as probiotic bacteria to formulate functional food.

**Authors' contributions**

DANGANG BOSSI Donald Sévérin, ZAMBOU Ngoufack François, AGRAWAL Renu and FONTEH Anyangwe Florence designed the study. Author DANGANG BOSSI Donald Sévérin performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ZAMBOU Ngoufack François and AGRAWAL Renu managed the analyses of the study. Author FONTEH Anyangwe Florence managed the literature searches. All authors read and approved the final manuscript.

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**Conflict of Interest:**

The authors declare that there are no conflicts of interests.

**Ethical approval**

The ethical guidelines are followed in the study for microbial experimentation.

**Data and materials availability:**

All data associated with this study are present in the paper.

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